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Inhibition of the Rous Sarcoma Virus Long Terminal Repeat-Driven Transcription by *in Vitro* Methylation: Different Sensitivity in Permissive Chicken Cells versus Mammalian Cells

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Rous sarcoma virus (RSV) enhancer sequences in the long terminal repeat (LTR) have previously been shown to be sensitive to CpG methylation. We report further that the high density methylation of the RSV LTR-driven chloramphenicol acetyltransferase reporter is needed for full transcriptional inhibition in chicken embryo fibroblasts and for suppression of tumorigenicity of the RSV proviral DNA in chickens. In nonpermissive mammalian cells, however, the low density methylation is sufficient for full inhibition. The time course of inhibition differs strikingly in avian and mammalian cells: although immediately inhibited in mammalian cells, the methylated RSV LTR-driven reporter is fully inhibited with a significant delay after transfection in avian cells. Moreover, transcriptional inhibition can be overridden by transfection with a high dose of the methylated reporter plasmid in chicken cells but not in hamster cells. The LTR, v-*src*, LTR proviral DNA is easily capable of inducing sarcomas in chickens but not in hamsters. In contrast, Moloney murine leukemia virus LTR-driven v-*src* induces sarcomas in hamsters with high incidence. Therefore, the repression of integrated RSV proviruses in rodent cells is directed against the LTR.

INTRODUCTION

Rous sarcoma virus (RSV) proviral DNA has previously been found to be heavily methylated at CpGs in RSV-infected but nontransformed rodent cells and in revertants that segregated spontaneously from RSV-transformed rodent cells (Hejnar *et al.*, 1994; Searle *et al.*, 1984). Some, but not all, of these proviruses can be activated by 5-azacytidine (5-azaC) (Searle *et al.*, 1984). An interesting situation was described in the rat tumor cell line XC (Svoboda, 1961), which contains highly amplified RSV genomes. These proviruses were found to be mostly hypermethylated (Guntaka *et al.*, 1980), but when rescued, they gave rise to unmethylated proviral copies in permissive chicken cells (Katz *et al.*, 1983). Avian endogenous proviruses (*ev* loci) display similar behavior: most of them are methylated and transcriptionally inactive, and some (e.g., RAV-0) are inducible by 5-azaC (Groudine *et al.*, 1981). Transcriptional suppression of methylated proviruses has also been described in cells infected by Moloney murine leukemia virus (MoMLV; Challita and Kohn, 1994; Hoeben *et al.*, 1991) and human immunodeficiency virus type 1 (HIV-1) (Bednarik *et al.*, 1990), but it is still unclear whether DNA methylation is a cause or merely a consequence of gene inactivation.

More specific effects of DNA methylation were shown in the case of HIV-1. Methylation at two discrete CpGs within the U3 region of HIV-1 LTR was sufficient for its inactivation, whereas coexpression of *tat* transactivator overrode this inactivation and removed methylation. These findings, supported by data obtained *in vivo*, suggest that methylation can play an important role in retroviral latency (Bednarik *et al.*, 1990; Gutekunst *et al.*, 1993; Schulze-Forster *et al.*, 1990). By analogy, *in vitro* methylation at CpGs at sites responsive to *tax* transactivator and phorbol myristate acetate (PMA) in the human T cell leukemia virus type I (HTLV-1) LTR abrogated expression from the LTR-driven reporter gene, whereas the coexpression of *tax* and concomitant stimulation with PMA removed methylation and restored expression (Saggioro *et al.*, 1991). Reversibility of the methylation suppression is, however, dependent on the density of methylation. When all CpGs in the HTLV-1 LTR were modified by *Sss*I methyltransferase, stimulation by *tax* and PMA was ineffective (Cassens *et al.*, 1994).

Direct and indirect molecular mechanisms by which DNA methylation controls the expression of cellular and proviral genes have recently been outlined. First, the presence of methylcytosine directly inhibits the sequence-specific binding of some protein transactivators, such as the cAMP-responsive element-binding protein

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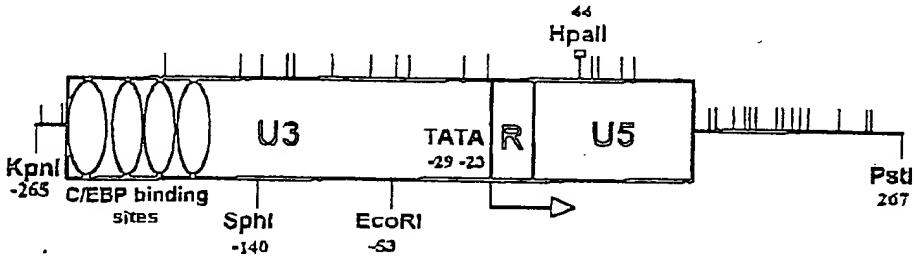

FIG. 1. Schematic representation of methylable sites in the Pr-RSV-C LTR and adjacent leader region present in the reporter plasmid pLTR-CAT. CpG dinucleotides are represented by vertical lines, and the single CCGG sequence (*HpaII* site) is denoted by open square. Four C/EBP binding sites in the enhancer portion of U3, TATA box, and transcription start (horizontal arrow) are displayed.

(Igouchi-Ariga and Schaffner, 1989). In contrast, transcription factors such as Sp1 and NF-1 are methylation resistant *in vitro* (Hoeller *et al.*, 1988), and at least some members of the consensus binding sequence bind Sp1 more strongly if they are methylated (Jane *et al.*, 1993). Second, methylated DNA can be bound by nuclear proteins, which secondarily prevent transcription factors from interacting with their binding sites. Among these proteins that bind preferentially to methylated DNA, the MDBP-2 protein is a member of the histone H1 family and acts as a high affinity repressor of the avian vitellogenin II gene promoter (Bruhat and Jost, 1995; Jost *et al.*, 1991). Histone H1 generally contributes to changes in chromatin structure and associates with methylated DNA regions (Lewis and Bird, 1991). Two other nuclear proteins, MeCP1 and MeCP2, are strong candidates as general methylation-dependent repressors due to their relaxed sequence specificity and widespread tissue distribution (Bird, 1992). MeCP1 binds to DNA containing at least 12 symmetrically methylated CpGs (Meehan *et al.*, 1989), and MeCP-2 requires a single methylated CpG pair (Lewis *et al.*, 1992). The transcriptional-repression domain of MeCP2 associates with a corepressor complex containing at least two histone deacetylases (Jones *et al.*, 1998; Nan *et al.*, 1998). Despite the fact that the LTR of murine myeloproliferative sarcoma virus belongs to the first promoter/enhancer sequences proved to be suppressed indirectly via MeCP (Boyes and Bird, 1991), little attention was paid to other retroviruses.

RSV LTR has previously been shown to be methylation sensitive by Guntaka *et al.* (1987). *In vitro* methylation of the U3 region by a mammalian liver methyltransferase decreased transcription of the LTR-driven *neo* gene, as measured by the *neo* mRNA level. In the present study, we quantified RSV LTR transcriptional activity more precisely using the chloramphenicol acetyltransferase (CAT) reporter at different densities of DNA methylation. We describe here different sensitivities to DNA methylation in chicken and hamster cells and propose that they might represent one of the mechanisms discriminating between permissive and nonpermissive cells.

RESULTS

High density DNA methylation inhibits RSV LTR-driven transcription

To estimate the effects of different density of DNA methylation on RSV LTR-driven transcription, we have transiently transfected 2 μg of *in vitro* methylated pLTR-CAT reporter DNA into chicken embryo fibroblasts (CEFs) and Syrian hamster cell line NIL-2, and assayed the resulting CAT activity 2 days after transfection. Different levels of DNA methylation were obtained using prokaryotic methylases *SssI*, *HpaII*, and *HhaI*. Representation of the RSV LTR with the respective methylation sites is shown in Fig. 1. CAT activity of the nonmethylated pLTR-CAT in CEFs was set as 100%. Methylation of all CpG sites by *SssI* methylase (modifies 16 CpGs throughout RSV LTR) inhibited transcription almost completely in both chicken and hamster cells. Low density methylation by *HpaII* methylase (modifies a single CpG within the U5 region of RSV LTR) resulted in a retention of 38% CAT activity of the nonmethylated control in CEFs but abrogated CAT activity in NIL-2 cells (Fig. 2). Methylation by *HhaI* methylase, which does not recognize any site within RSV LTR, produced barely detectable effects on CAT activity both in CEFs and in NIL-2 cells. Thus, RSV LTR is extremely sensitive to low density methylation in mammalian NIL-2 cells and requires high density methylation to be inactivated in permissive chicken cells. For comparison, we also performed these experiments with QT6, NIH-3T3, and LWEF cells and observed similar sensitivity to low density methylation in mouse NIH-3T3 cells and rat LWEF cells. QT6 cells resembled CEFs with the exception of weaker inhibition of densely methylated plasmid DNA; this might be caused by some transcriptional dysregulation accompanying the transformed state of this cell line. In one experiment, we eliminated the adjacent vector DNA whose methylation might contribute to the total inactivating effect. We used *Hin*dIII and *Bam*HI restriction endonucleases with recognition sites upstream of the RSV LTR and downstream of the SV40 poly(A)$^+$, respectively, leaving the reporter proviral structure within a 2.7-kb DNA fragment *Bam*HI-*Hin*dIII dou-

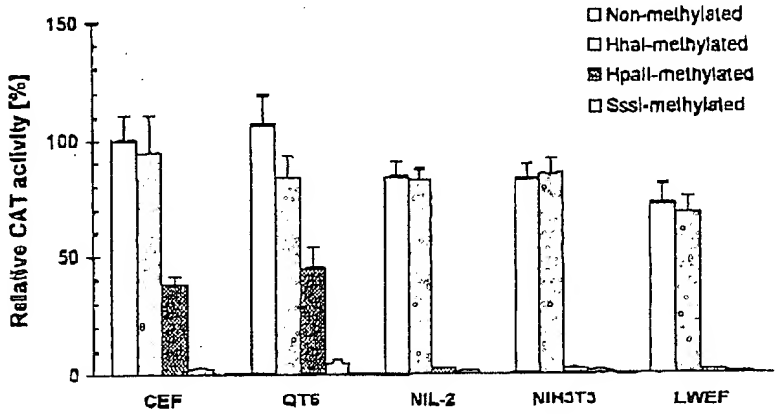

FIG. 2. Inhibition of the RSV LTR-driven transcription by DNA methylation. CEFs, QT6, NIL-2, NIH-3T3, and LWEF cells were transiently transfected with 2 μg of the pLTR-CAT DNA methylated *in vitro* by *Sss*I, *Hha*I, and *Hpa*II methyltransferases. As a control, nonmethylated DNA was used. CAT activity was measured 48 h posttransfection. The CAT activity of nonmethylated reporter DNA in CEFs is calculated as 100%. This experiment was repeated independently three times. Data of one representative experiment performed in triplicate are presented as mean ± SEM values.

ble-digested pLTR-CAT DNA was methylated by *Sss*I or *Hpa*II methylases and used for CAT assay in CEFs and NIL-2 cells. CAT activities in this experiment were not significantly higher than that of nondigested reporter DNA (data not shown) with the exception of *Hpa*II-methylated DNA in CEFs (but not in NIL-2 cells). CAT activity here reached 53% of the nonmethylated control.

Time course of methylated RSV LTR inactivation after transfection

To gain some understanding of the mechanism that brings about the inhibition of RSV LTR-driven transcription by DNA methylation, we measured the CAT activity of *in vitro* methylated pLTR-CAT on 3 consecutive days posttransfection. Figure 3A shows that in CEFs and QT6 cells, the transcriptional inhibition of *Sss*I-methylated pLTR-CAT increases with time, being relatively weak the first day posttransfection (20% and 34% of the CAT activity of the nonmethylated control) and reaching a maximum on the third day. In contrast, expression of the *Hpa*II-methylated pLTR-CAT increases slowly, almost reaching the expression of the nonmethylated control on the third day posttransfection.

Maintenance of the *in vitro* DNA methylation after transfection into cells is shown in Fig. 4. We transfected in parallel nonmethylated (lanes 2 and 3), *Hpa*II-methylated (lanes 4 and 5), and *Sss*I-methylated (lanes 6 and 7) plasmid pLTR-CAT into CEFs and isolated the total cellular DNA 3 days posttransfection. These DNAs were cleaved separately by *Hpa*II (lanes 3, 5, and 7) and *Msp*I (lanes 2, 4, and 6). Southern blot hybridized with LTR and leader probe shows that the *Msp*I-produced bands of 416 and 932 bp are absent in *Hpa*II digests (lanes 5 and 7) and indicates that DNA methylation of 5′-CCGG-3′ sequences in U5 region and adjacent vector DNA is kept during the transient transfection period. It is, however, not clear whether the overall CpG methylation along the reporter construct is maintained precisely during this time. Also, a longer retention of methylated plasmid in the cell nuclei was described (Hsieh, 1994). Expression of *Sss*I-methylated DNA soon after transfection indicates that at least some of the LTR-binding transcription factors are not sensitive to CpG methylation *per se* and that rather slowly acting indirect mechanisms (i.e., binding of a protein repressor and/or changes in chromatin conformation (Jones *et al.*, 1998; Nan *et al.*, 1998) might be involved. Such a delay in transcriptional repression has been described after microinjection of methylated herpes simplex thymidine kinase DNA into cell nuclei (Buschhausen *et al.*, 1987). Again, RSV LTR in NIL-2 and NIH-3T3 cells is more sensitive to both high and low density methylation than in CEFs or QT6 because it is fully inactivated as soon as 24 h after transfection (Fig. 3B).

Transfection of high doses of DNA overrides the methylation-caused inhibition

Another evidence that the RSV LTR is more sensitive to DNA methylation in mammalian than in avian cells comes from the transfection assay with high doses of *Sss*I-methylated pLTR-CAT (Fig. 5). Transfection with 0.5–2.0 μg of methylated DNA resulted in barely detectable CAT activity, reaching 2–3% of that of the nonmethylated DNA. Transcriptional capacity of chicken cells seemed to be fully saturated with 2.0 μg of nonmethylated pLTR-CAT DNA because transient transfection of 5 μg plasmid DNA did not lead to a significant increase in the CAT activity. Transfection with 5.0 μg of *Sss*I-methylated DNA, however, overrode the transcriptional inhibition with 34% of CAT activity compared with the nonmethylated control (Fig. 5A). In contrast to CEFs, high doses of *in vitro* methylated DNA are fully inactive after transfection into NIL-2 cells (Fig. 5B). In a parallel experiment, QT6 and NIH-3T3 cells resemble CEFs and NIL-2 cells, respectively (data not shown). These results suggest again that

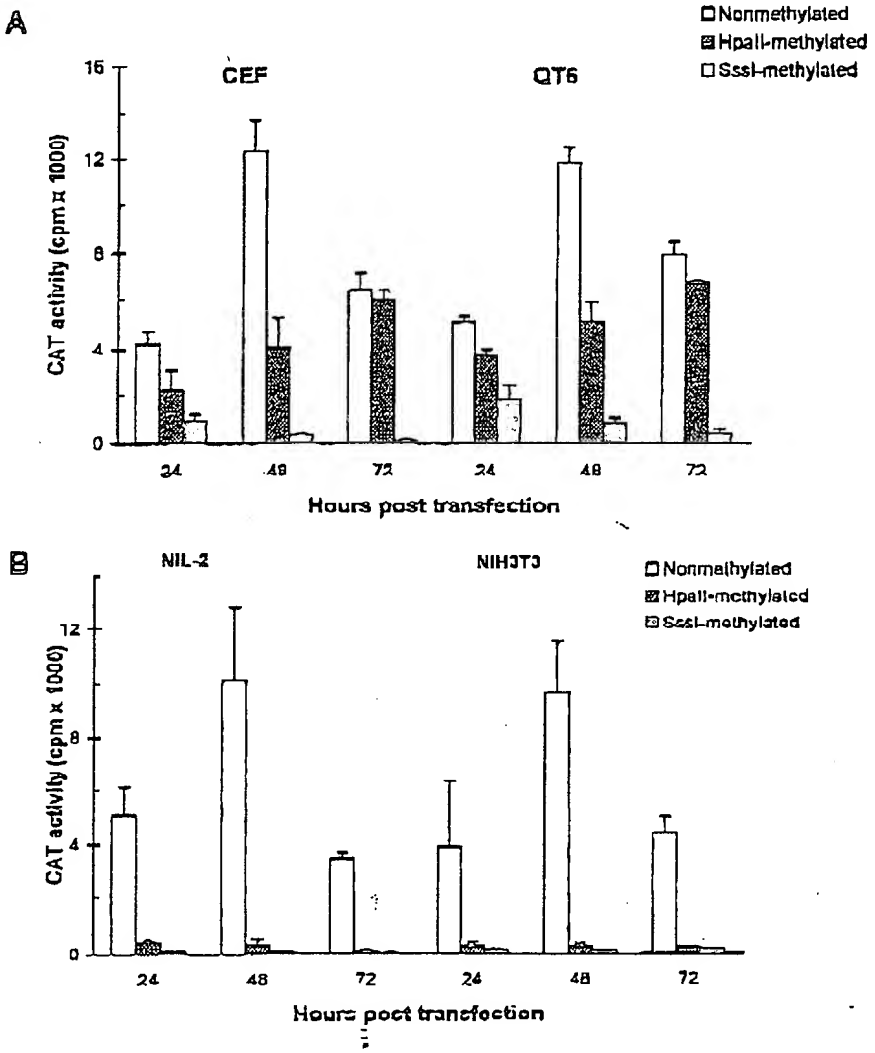

FIG. 3. Time course of the transcriptional inhibition of the methylated pLTR-CAT DNA after transfection in CEFs and QT6 (A) or NIL-2 and NIH-3T3 cells (B). Cultivated cells were transfected with 2 μg of *Sssl*-methylated, *Hpa*II-methylated, and nonmethylated plasmid DNA and the CAT activity was measured 24, 48, and 72 h posttransfection. These results represent the mean ± SEM from two independent experiments performed in duplicate.

mammalian cells are more capable of inactivating methylated RSV LTR than permissive chicken cells and excludes a possibility that reasonable CAT activity after transfection of high dose of the reporter plasmid into CEFs is caused by nonmethylated fraction of the plasmid DNA. This saturation together with the time course of inhibition in permissive cells indicates again that methylation of the RSV LTR alone does not simply mask the recognition sites of sequence-specific transcriptional activators. Levine *et al.* (1991) described similar overriding of the methylation-dependent inhibition of mouse metallothionein I gene promoter as an exhaustion of methylcytosine-binding *trans* repressors. Indeed, some of the methylation repressors might be of relatively low abundance (e.g., ~5000 molecules of MeCP1 per nucleus, Meehan *et al.*, 1992). Under such conditions, overriding the inhibition by cotransfection of the reporter plasmid with the excess of a nonspecific methylated competitor might be possible. However, our preliminary experiments aimed to show such a competition was unsuccessful (data not shown), which suggests that conditions differ between the models of Levine *et al.* and our experimental models.

Methylated RSV proviral DNA does not induce progressively growing sarcomas in chickens

To evaluate whether DNA methylation of RSV LTR is sufficient for provirus inactivation, we tested the tumor-forming capacity of *in vitro* methylated cloned proviral DNA. Previously, we have shown that linearized cloned DNA of the LTR, v-*src*, LTR provirus induced sarcomas in chickens with high efficiency and short latency after subcutaneous inoculation (Plachý *et al.*, 1994; Svoboda *et al.*, 1992). One-day-old chicks of the Prague inbred line PR-CC.R1 (Plachý *et al.*, 1989) were inoculated sepa-

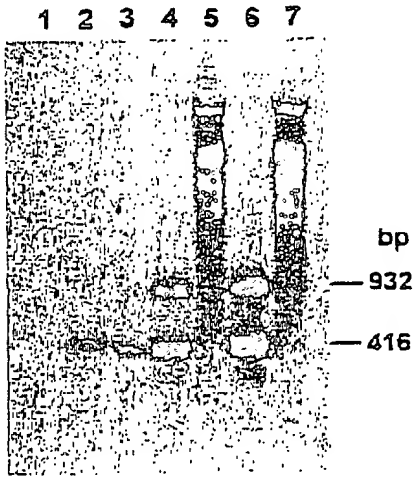

FIG. 4. Maintenance of *in vitro* DNA methylation within the LTR of the transfected plasmid DNA. Nonmethylated (lanes 2 and 3), *Hpa*II-methylated (lanes 4 and 5), and *Sss*I-methylated (lanes 6 and 7) pLTR-CAT plasmid DNA was transfected into CEFs and isolated 3 days posttransfection. DNA samples were cleft separately by *Msp*I (lanes 2, 4, and 6) and *Hpa*II (lanes 3, 5, and 7), migrated in 1% agarose gel, blotted onto nylon membrane, and hybridized with a radioactively labeled *Kpn*I–*Pst*I fragment of RSV LTR and leader (Fig. 1). *Msp*I-cleaved DNA from nontransfected CEFs (lane1) was used as a control. *Msp*I produces bands of 932 bp (U3, part of U5 and 5′ adjacent part of the vector sequences) and 416 bp (part of U5, leader and 3′ adjacent part of the CAT gene). *Hpa*II produces bands of clearly higher molecular weight in both *Hpa*II- and *Sss*I-methylated reporter plasmid.

rately with 0.5 μg of *Sss*I-methylated, *Hpa*II-methylated, and nonmethylated proviral DNA, and tumor occurrence and growth were monitored for 60 days postinoculation. LTR, v-*src*, LTR DNA used was the pH-19r1cl.3 linearized by *Hin*dIII cleavage (Hejnar *et al.*, 1994). Nonmethylated DNA induced progressively growing sarcomas in all inoculated animals as soon as 12–21 days postinoculation. *Hpa*II-methylated DNA induced sarcomas also with 100% efficiency but with a slightly longer latency and with a clear tendency to regression. High density methylation by *Sss*I reduced the incidence of sarcomas to 30%. Almost nonpalpable tumors appeared after a latency of 28–43 days and regressed completely after 2–4 weeks of persistence. The kinetics of tumor growth are shown in Fig. 6.

MoMLV LTR-, but not RSV LTR-, driven v-*src* gene induces sarcomas in hamsters

In accordance with the low efficiency of the transformation of rodent cells by RSV, we expected that tumor induction by v-*src* gene DNA under the control of RSV LTR could be very exceptional in the Syrian hamster. We inoculated newborn hamsters with 1 μg or 10 μg of pH-19r1cl.3 plasmid DNA linearized by *Hin*dIII endonuclease and inspected the tumor occurrence for 18 months. During this time, no tumor appeared at the site of inoculation (Table 1), although the higher dose of plasmid DNA represents 100-fold excess of DNA inducing sarcomas nearly in 100% of the inoculated chicks (J. Plachý and J. Hejnar, unpublished data).

To obtain v-*src* DNA-induced tumors in hamsters, we tried to promote tumor induction by an antimethylation treatment of inoculated animals because demethylation of inactive proviruses often led to cell transformation. Another cohort of 54 newborn hamsters from 8 litters was inoculated with a high dose of linearized pH-19r1cl.3 DNA and injected weekly with 5 μg of 5-aza-dC/5 g body weight. Injections of 5-azadC were started on day 7 and continued for 100 consecutive days. Animals were monitored for 20 months, but only one sarcoma at the site of inoculation appeared during this time. DNA analysis of this sarcoma by Southern blotting did not show the presence of any part of the proviral structure (data not shown), and therefore induction of this tumor should be attributed to the mutagenic effects of 5-aza-dC alone rather than to the proviral activity (Table 1).

Although the lack of tumor induction in hamsters fits well with the sensitivity of RSV LTR to DNA methylation described in transient transfection experiments, it was not clear whether the proviral DNA was inactivated efficiently after integration into the hamster genome or, alternatively, the technique of DNA inoculation was not proper for the delivery of proviral DNA into the target cell. An indication that the v-*src* gene DNA is potentially capable of sarcoma induction *in vivo* came from experiments with MoMLV LTR-driven v-*src*. Circular pMvsrc plasmid DNA was used due to the scarcity of restriction sites outside the coding or regulatory regions. Newborn hamsters were routinely inoculated with 1 or 5 μg of plasmid DNA without any antimethylation treatment and monitored for 8 months. In this experiment, sarcomas were induced in 69% of the inoculated animals after 13–54 days (mean latency, 28 days). These sarcomas varied with respect to progressive growth, persistence, or regression. *Sss*I-methylated pMvsrc DNA failed to form sarcomas, as well as the *Pvu*II-digested pMvsrc DNA cleaved three times within the v-*src* coding sequence (Table 1). The presence of the v-*src* gene in hamster sarcomas was evidenced by PCR. Figure 7 shows two representative progressively growing v-*src*-positive pMvsrc-induced sarcomas. These results not only demonstrate the efficiency of sarcoma induction by v-*src* but indicate that the type of LTR is critical for provirus activity or silencing and that the RSV LTR might be the principal target for inactivation by methylation.

DISCUSSION

Our study describes different levels of transcriptional suppression of methylated RSV LTR in permissive chicken cells and nonpermissive hamster cells in the transient assay (i.e., before integration into the host genome). After integration, the methylated RSV LTR might

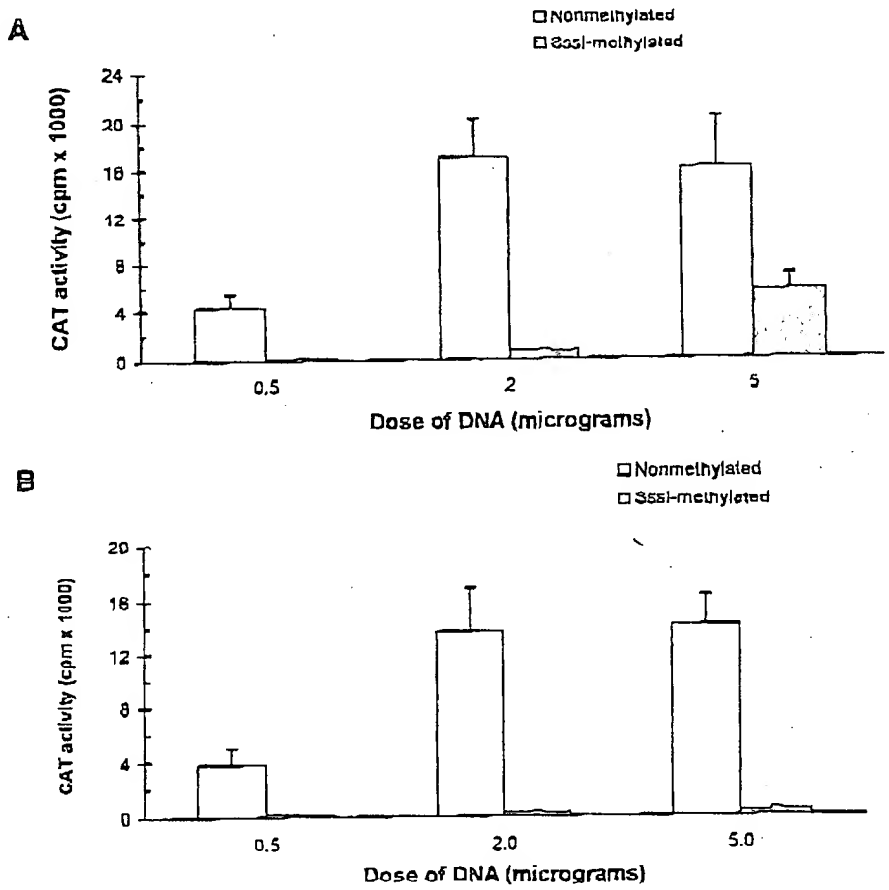

FIG. 5. Transcriptional inhibition of *Sssl*-methylated pLTR-CAT after transfection with high doses of DNA. CEFs (A) and NIL-2 cells (B) were transfected with increasing doses of methylated DNA and corresponding doses of nonmethylated DNA were used as controls. CAT activity was measured 2 days posttransfection in a single experiment performed in triplicate and the results are presented as mean ± SEM values.

be subverted to further inactivating events. In mammalian cells, RSV LTR-driven proviruses or constructs are transiently active (Machoń *et al.*, 1996; Overbeek *et al.*, 1986) but are rapidly methylated and inactivated after integration, with the exception of those fortuitously integrated in the vicinity of CpG islands (Fincham and Wyke, 1991). It is not the case of chicken cells, where exogenously introduced RSV proviral copies are not detectably methylated over the background of methylated endogenous *ev* loci (Guntaka *et al.*, 1980; Katz *et al.*, 1983). Clonal analysis of chicken cells lacking endogenous proviral copies infected with replication defective virus or RSV LTR-driven reporter could unambiguously solve this question. However, we show that *in vitro* methylated proviral DNA introduced into chicken cells *in vivo* obviously does not induce progressively growing sarcomas, which means that the integrated proviruses are kept inactive, probably methylated.

The sensitivity of RSV LTR to DNA methylation within the U3 region was described previously by Guntaka *et al.* (1987) in quail QT6 cells using the rat liver methyltransferase. To assess more precisely the degree of this down-regulation, we measured the transient expression of the RSV LTR-driven CAT reporter construct methylated *in vitro* by defined prokaryotic methyltranferases *Sssl*, *Hpa*II, and *Hha*I in CEFs. In contrast to the experiments of Guntaka *et al.*, we methylated not only the LTR but also the CAT coding sequence and flanking vector DNA. No significant effect of *Hpa*II or *Sssl* methylation in the coding region of the CAT gene has previously been documented (Rosl *et al.*, 1993; Schroeder and Mass, 1997). Also, in other reporter genes, such as luciferase or β-galactosidase genes, *Sssl* methylation of the coding sequences was not sufficient for full transcriptional suppression (Cassens *et al.*, 1994; Muiznieks and Doerfler, 1994). In addition to the RSV LTR and CAT coding sequence, the GC-rich prokaryotic vector DNA was methylated in our experiments. Although many authors failed to observe any significant effects of methylated flanking DNA, Bryans *et al.* (1992) described inhibition of transcription from the SV40 early promoter by vector *Sssl* methylation. Similarly, Pichon *et al.* (1994) described repression of the unmethylated thyroglobulin promoter by methylation of flanking plasmid DNA. To determine whether methylation of vector DNA contributed to the inhibition effect, we used a double-digested reporter

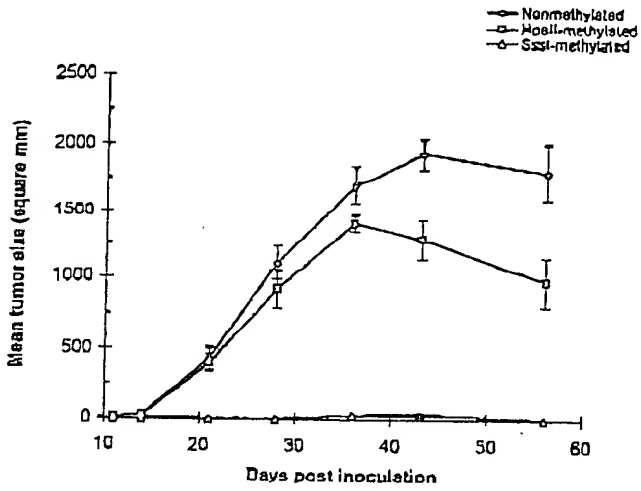

FIG. 6. Kinetics of tumor growth induced by 0.5 μg of *Sssl*-methylated, *Hpa*II-methylated, and nonmethylated LTR, v-*src*, LTR DNA in chickens. The average size of tumors (in mm^2) ± SEM was calculated from the individual values of all nine chicks in each group in a given period. Chickens that had regressed their tumors completely were included in the calculation with the zero value.

TABLE 1

Tumor Induction by v-*src*-Containing Proviral DNA in Newborn Hamsters

Inoculum (plasmid digestion)	Frequency of hamsters with sarcomas[a]	Late (da
pH19r1cl.3 1 μg(*Hin*dIII)	0/67 (0%)	
pH19r1cL3 10 μg (*Hin*dIII)	0/39 (0%)	
pH19r1cl.3 50 μg (*Hin*dIII)	0/6 (0%)	
pH19r1cl.3 10μg (*Hin*dIII) + 5-aza-dC	1/54 (2%)[b]	35
pMvsrc 1 μg (nondigested)	15/22 (68%)	13-
pMvsrc 5 μg (nondigested)	3/4 (75%)	1
pMvsrc 1 μg (nondig., *Sss*I methylated)	0/8 (0%)	
pMvsrc 5 μg (*Pvu*II)	0/5 (0%)	

[a] Frequency of hamsters with sarcomas at the site of inoculatic expressed as the number of hamsters developing sarcomas/numb hamsters inoculated.

[b] This sarcoma cannot be regarded as v-*src*-induced because DNA analysis did not show the presence of either v-*src* gene or sequences.

construct with eliminated flanking vector sequences and found that the methylated DNA was transcriptionally repressed to a similar degree as the whole methylated reporter construct. Taken together, we conclude that the main characteristics of the methylation-dependent inactivation of the RSV LTR-driven reporter can be attributed to the RSV LTR with only minor and nonsignificant contributions by the remainder of the reporter construct.

It is difficult to explain how the *Hpa*II methylation of a single CpG out of the promoter or enhancer region of the LTR can effectively suppress transcription; similar site-specific effects of methylation on HIV-1 or HTLV-1 LTR map near to the nuclear factor-κB- and Sp1-binding sites or Tax- and PMA-responsive element, respectively (Bednarik *et al.*, 1990; Saggioro *et al.*, 1991). Recently, however, the importance of sequences located downstream of the HIV-1 transcription start within the U5 and leader region has been recognized for proviral transcription activity and chromatin structure (El Kharroubi and Martin, 1996; Van Lint *et al.*, 1997). In our reporter construct, there is a cluster of 14 CpGs without *Hpa*II site in the RSV leader region (Fig.1).

DNA methylation of proviral sequences has usually been regarded as a passive and secondary event in the transcriptional inactivation of retroviruses, as an additional lock mechanism against reactivation of previously inactivated proviral copies. However, methylation in the promoter/enhancer regions has been shown to be crucial for suppression of provirus or gene expression, whereas methylation within the open reading frames or 3′ to them has little effect (Cassens *et al.*, 1994; Guntaka *et al.*, 1987). Other data also suggest that DNA methylation plays a more active role in the virus–cell interaction. For example, Joel *et al.* (1993) described a nucl protein with enhanced binding to methylated Sp1 sites the LTR of HIV-1 distinct from all so far known prote with affinity for methylated DNA. This protein, HI methylated DNA binding protein (HMBP), is expressed human $CD4^+$ T cells and might be a prototype of cellu proteins, bringing about the retroviral latency via D methylation. In contrast, frog virus 3 (FV3) is a cl

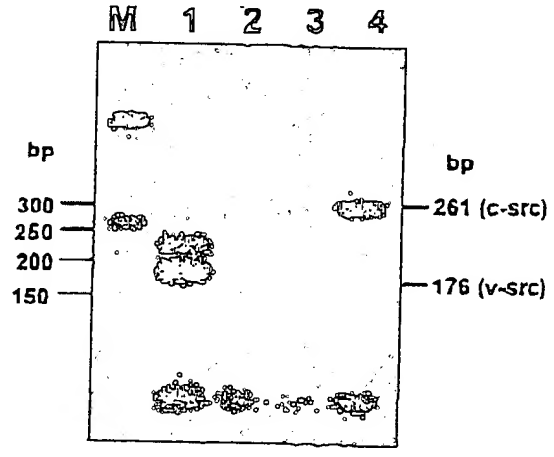

FIG. 7. PCR detection of v-*src* gene in DNAs from pMvsrc-indu tumors. DNAs isolated from sarcomas pMvsrc6/6 and pMvsrc9/6 gether with negative and positive controls were subverted to PCR described under "Materials and Methods." Products of PCR were s fractionated in agarose gel and visualized by ethidium bromide sta ing. Lane M indicates 50-bp ladder (Boehringer Mannheim); lane 1, L v-*src*, LTR-transformed chicken tumor cell line 9692 (Svoboda *et* 1992) displaying a v-*src*-specific 176-bp fragment used as a posi control; lane 2, sarcoma pMvsrc6/6; lane 3, sarcoma pMvsrc9/6; lane 4, uninfected CEFs displaying a c-*src*-specific 261-bp fragm used as a negative control.

example of a viral antimethylation strategy. FV3 encodes its own methyltransferase to protect the viral genome DNA from endonucleolytic cleavage and concomitantly, a so-far-unidentified protein encoded by FV3 promotes the expression of heavily methylated viral and foreign DNA (Spangler and Essani, 1994). Finally, a further understanding of proviral inactivation by DNA methylation might shed new light on retroviral latency as well as the host permissiveness to retroviruses.

Based on the fact that the large majority of methylcytosine in the mammalian genome comes from parasitic sequences that arose by retrotransposition, many authors suggest that DNA methylation in vertebrates gradually evolved as a host defense system against these genome parasites (reviewed by Doerfler, 1991; Yoder *et al.*, 1997). Antimethylation strategies are therefore an important part of the coevolution of retroviruses and their hosts. For example, comparison of the content of CpG dinucleotides in genomes of HIV-1 ($CpG_{expected/observed}$ ratio, 4.92) and HIV-2 strains ($CpG_{expected/observed}$ ratio, 2.98) shows that HIV-1 probably is evolving to remove host-methylable CpGs from its genome (Nyce, 1996). This correlates with the different virulence of these two viruses.

In summary, we have shown that the transient RSV LTR-driven expression is more efficiently suppressed by methylation in nonpermissive rodent cells than in permissive chicken cells. Our *in vivo* sarcoma induction experiments suggest that after integration into rodent cells, RSV proviruses are inactivated by a general suppressive mechanism directed against the RSV LTR but not MoMLV LTR. All these data point to proviral DNA methylation as an important event in the nonpermissiveness to retroviral expression and retroviral latency. Furthermore, avoidance of this hindrance can lead to the construction of RSV-based retroviral vector for mammalian cells. Mechanisms of RSV LTR inactivation and antimethylation strategies are under investigation.

MATERIALS AND METHODS

Plasmid constructs

Reporter plasmid pLTR-CAT was constructed by insertion of *KpnI–PstI* fragment from the cloned provirus H-19 (RSV LTR and leader sequences, nucleotides 504-1037 according to Bodor *et al.*, 1989) into the polycloning site of pCAT-Basic expression vector (Promega) containing the promoter-less chloramphenicol acetyltransferase (CAT) gene coding sequence and SV40 poly(A)$^+$ (Machoň *et al.*, 1996). Plasmid pH-19r1cl.3 contains the LTR, *v-src*, LTR proviral structure flanked by adjacent sequences of hamster DNA inserted into the *HindIII* site of the vector pGEM4Z (Promega). Its construction and biological activity were described previously (Hejnar *et al.*, 1994). Plasmid pMvsrc (a kind gift of Dr. M. S. Halpern) contains the Schmidt Ruppin A *v-src* gene together wi *Δenv* flanked by MoMLV LTRs (Johnson *et al.*, 1985).

Cells and cell culture

CEFs were prepared from 10-day brown leghorn en bryos, phenotype C/E, by standard procedures. Japanes quail (*Coturnix coturnix japonica*) fibrosarcoma cell lin QT6 (Moscovici *et al.*, 1977) was used in parallel to CEFs The immortalized Syrian hamster fibroblastoid cell lin NIL-2 was developed by Diamond (1967). The mous NIH-3T3 cell line and Lewis rat immortalized fibroblas cell line LWEF were used in several experiments ir parallel to NIL-2. Cells were routinely grown in the mix c two parts Ham's F10 and one part DMEM supplemente with 5% calf serum, 5% FCS, and penicillin/streptomycir in a 5% CO_2 atmosphere at 37°C and passaged twice weekly. CEFs and QT6 cells were further supplemente with 1% chicken serum.

In vitro DNA methylation

Plasmid DNA was methylated *in vitro* by prokaryotic methylases *HpaII* (MBI-Fermentas), *HhaI*, or *SssI* (both New England Biolabs) for 2–4 h at 37°C using 1 U of enzyme/μg of DNA in buffers supplied by the manufacturer. Then, 80 or 160 μM *S*-adenosylmethionine was added to *HpaII* and *HhaI* or *SssI* methylases, respectively. In mock-methylation reactions, enzyme was omitted. To avoid incomplete methylation, entire products of methylation reactions were digested for 1 h by restriction endonucleases *HpaII* or *HhaI* (1 U/μg DNA), and aliquots were analyzed by agarose gel electophoresis. Only completely methylated samples were used for experiments.

Confirmation of the DNA methylation after transient transfection

To confirm the degree of DNA methylation of *in vitro* methylated plasmids during transient transfection experiments, we isolated the total DNA from transfected cultures 3 days posttransfection and analyzed the DNA methylation within 5′-CCGG-3′ sequences by Southern blotting. Total DNAs were isolated using the silica-gel membrane column (QIAamp tissue kit; Qiagen) to avoid the loss of small plasmid molecules during alcohol precipitation. DNA samples were in parallel cleaved by *HpaII* and *MspI* restriction endonucleases (Promega), migrated in 1% agarose gel, and blotted onto nylon membrane (Zeta Probe; Bio-Rad). Southern blots were hybridized with the radioactively labeled *KpnI–PstI* fragment comprising the RSV LTR and leader sequences cloned into pLTR-CAT reporter plasmid (Fig.1).

Transient transfection

Transient transfection experiments were performed on 60-mm dishes seeded with 0.75×10^6 primary CEFs (or

0.5×10^6 QT6, NIL-2, NIH-3T3, and LWEF cells) 20–24 h before transfection. Transfection (lipofection) was performed using 20 μl of *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methylsulfate transfection reagent (Boehringer Mannheim) according to the manufacturer's instructions in serum-free medium. After 8 h of incubation, the cells were washed and then cultured in medium supplemented with serum as indicated. The efficiency of transfection was found to be reproducible without significant variations using a standard β-gal assay (not shown). We also found minimal variance between parallel samples.

CAT assays

CAT assay was performed essentially according to Seed and Sheen (1988) with some modifications. Briefly: transfected cells were harvested and disrupted by three cycles of freezing in dry ice and thawing at 37°C in 0.25 M Tris–Cl, pH 7.5. Then, 10 μl of the supernatant was incubated in 100 μl of 125 mM Tris–Cl, pH 7.5, 0.25 mM *n*-butyryl-coenzyme A (Sigma), and 0.1 μCi of ^{14}C-chloramphenicol (Amersham, 55 mCi/mmol) for 1 h at 37°C. The reaction was stopped by adding 0.2 ml of xylene, vortexing, and centrifugation for 2 min at 13,000 rpm, and the upper organic phase containing extracted butyrylated chloramphenicol was reextracted against 0.1 ml of TE (10 mM Tris–Cl, pH 7.6, 1 mM EDTA) and measured in a liquid scintillator. These results were finally normalized to the protein concentration of supernatants estimated by the Bradford dye binding method using the Protein Assay (Bio-Rad).

Animals, DNA inoculation, and 5-aza-dC treatment

The animals used in sarcoma-induction experiments were bred at the Institute of Molecular Genetics (Prague). Avian leukosis-free chickens of the inbred line PR-CC.R1 have been classified previously as progressors of v-*src*-induced tumors (Plachý *et al.*, 1989; Svoboda *et al.*, 1992). Syrian hamsters (*Mesocricetus auratus*) have been inbred for >40 generations by brother × sister matings and characterized by the acceptance of skin grafts.

Plasmid DNAs were purified on a Qiagen column 500 and linearized outside of the proviral sequences. The digests were diluted in PBS A and inoculated in 0.1 ml usually containing 1 μg of plasmid DNA. For comparison, the proviral inserts represent ~50% of the whole plasmid. Nine- to 10-day-old chickens were inoculated subcutaneously through the pectoral muscle, and the growth of sarcomas was monitored by estimating the areas of tumors (Svoboda *et al.*, 1992). Newborn hamsters were inoculated subcutaneously, and the incidence and latency of sarcoma induction were scored.

5-Aza-dC injections were performed according to Laird *et al.* (1995), whose technique led to a reduced DNA methyltransferase activity *in vivo* and to creased level of genomic DNA methylation. The 5-a (Sigma) was diluted in proper concentration in and stored at −70°C. Animals were weighed a jected subcutaneously with 5 μg of 5-aza-dC/5 g o weight in 0.1–0.2 ml of PBS A. Injections were perf weekly starting at 7 days of age and continued additional 100 days (14 injections). Control animal injected with PBS A alone.

PCR detection of v-*src* gene in DNA-induced sarcomas

For detection of v-*src* gene in the DNA isolate pMvsrc-induced hamster sarcomas, we used PCR ers residing in the v-*src* coding region. Sequ are 5′-CTGCTTTGGAGAGGTCTGGA-3′ and 5′-T GGGCTCTTCCGACAC-3′ corresponding to the n tides 7956–7975 and complementarily to the nucle 8113–8132 of the Pr-C RSV, respectively (Schwartz 1983). The expected fragment of 176 bp was amplif 2.5 U of *Taq* polymerase (Boehringer Mannheim 25-μl reaction mixture containing 1× buffer (Boeh Mannheim), 2.5 mM $MgCl_2$, 200 nM dNTP mix, 150 each primer, and 300 ng of template DNA isolate the sarcomas. The first five cycles of amplificati cluded a 1-min denaturation at 95°C, 1.5-min ann at 63°C, and 2-min extension at 72°C. In an additio cycles, both annealing and extension times were ened to 1 min at 63°C and 72°C, respectively.

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